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A POLYMORPHISM OF A PLATELET GLYCOPROTEIN RECEPTOR AS AN INHERITED RISK FACTOR FOR CORONARY THROMBOSIS

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Abstract *Background.* Platelet glycoprotein IIb/IIIa is a membrane receptor for fibrinogen and von Willebrand factor, and it has an important role in platelet aggregation. It is known to be involved in the pathogenesis of acute coronary syndromes. Previously, we found a high frequency of a particular polymorphism, Pl^{A2}, of the gene encoding glycoprotein IIIa in kindreds with a high prevalence of premature myocardial infarction.

Methods. To investigate the relation between the Pl^{A2} polymorphism and acute coronary syndromes, we conducted a case–control study of 71 case patients with myocardial infarction or unstable angina and 68 inpatient controls without known heart disease. The groups were matched for age, race, and sex. We used two methods to determine the Pl^A genotype: reverse dot blot hybridization and allele-specific restriction digestion.

Results. The prevalence of Pl^{A2} was 2.1 times higher among the case patients than among the controls (39.4 percent vs. 19.1 percent, P = 0.01). In a subgroup of patients whose disease began before the age of 60 years, the prevalence of Pl^{A2} was 50 percent, a value that was 3.6 times that among control subjects under 60 years of age (13.9 percent, P = 0.002). Among subjects with the Pl^{A2} polymorphism, the odds ratio for having a coronary event was 2.8 (95 percent confidence interval, 1.2 to 6.4). In the patients less than 60 years of age at the onset of disease, the odds ratio was 6.2 (95 percent confidence interval, 1.8 to 22.4).

Conclusions. We observed a strong association between the Pl^{A2} polymorphism of the glycoprotein IIIa gene and acute coronary thrombosis, and this association was strongest in patients who had had coronary events before the age of 60 years. (N Engl J Med 1996;334:1090-4.)

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S TUDIES indicate that myocardial infarction and unstable angina result from the formation of a platelet aggregate at the site of a ruptured coronary atherosclerotic plaque.[1-7] The formation of such aggregates requires the binding of fibrinogen and von Willebrand factor to the receptor, glycoprotein IIb/IIIa, on the platelet surface.[6,8,9] Large trials have demonstrated a marked benefit of various inhibitors of platelet function in both preventing and reducing the mortality and morbidity associated with unstable coronary syndromes.[10-26] Additional studies have linked ex vivo platelet reactivity to outcome in patients after myocardial infarction.[27] In sum, there is strong evidence that platelets, and glycoprotein IIb/IIIa in particular, have an important role in the pathogenesis of acute coronary syndromes.

Platelet-membrane glycoproteins are highly polymorphic and can be recognized as alloantigens or autoantigens.[28] The alloimmune thrombocytopenias are due to the incompatibility of epitopes on the various platelet-surface glycoproteins.[29] The alloantigen referred to as Pl^A or Zw is the one most frequently implicated in syndromes of immune-mediated platelet destruction.[30,31] Kunicki and Aster demonstrated that anti-Pl^{A1} antiserum from Pl^{A1}-negative persons reacted with platelet glycoprotein IIIa.[32] Subsequently, Newman et al. identified the molecular basis of this polymorphism: persons positive for Pl^{A1} have a leucine at position 33 of mature glycoprotein IIIa; persons positive for Pl^{A2} have a proline at this position, which is the result of the substitution of cytosine for thymidine at position 1565 in exon 2 of the glycoprotein IIIa gene.[33] We recently determined the allelic frequencies of Pl^{A1} and Pl^{A2} in several racial and ethnic groups in the metropolitan Baltimore area.[34]

Previously, we found an unexpectedly high frequency of family members homozygous for Pl^{A2} in kindreds with a high prevalence of acute coronary events at a relatively young age (under 60 years).[35] This observation led us to postulate that the presence of at least one Pl^{A2} allele may be related to the development of symptomatic unstable coronary heart disease. We conducted a case–control study to examine whether there was an association between the Pl^{A2} allele and unstable coronary syndromes, especially in persons less than 60 years of age.

METHODS

Selection of Case Patients and Control Subjects

Studies on case patients and control subjects were approved by the Joint Committee for Clinical Investigation of Johns Hopkins University and Hospital. Genotypic analyses were performed on 71 consecutive case patients admitted to the Coronary Care Unit of Johns Hopkins Hospital with an established diagnosis of myocardial infarction or unstable angina as defined by World Health Organization criteria. Similar genotypic analyses were performed on control subjects matched with the case patients for age, race, and sex, but who had no documented history of either stable or unstable angina or myocardial infarction. The control subjects were selected by reviewing patient charts from a population of patients admitted to the general medical

and intensive care services of the hospital. We selected 68 consecutive subjects who fulfilled these criteria.

Demographic Characteristics

Demographic data were obtained on each subject from the official medical record at the time of enrollment and included the current age (for control subjects), the age at the time of a first event (for case patients), sex, smoking history, blood pressure, total serum cholesterol level, diabetes status, and history of coronary events. Because the prevalence of the *Pl^A2* allele is known to be lower among blacks than among whites, we limited the study to the white population.[34,36]

Determination of *Pl^A* Genotypes

Genomic DNA was isolated from 200 μl of whole blood as previously described or with the QIAamp blood kit (Qiagen, Chatsworth, Calif.).[37] To detect the substitution of cytosine for thymidine responsible for the *Pl^A2* polymorphism at position 1565 in exon 2 of the glycoprotein IIIa gene, we used both reverse dot blot hybridization and allele-specific restriction digestion (exon numbering and nucleotide sequence are from Zimrin et al.[38]). Exon 2 was amplified from genomic DNA from case patients or controls in a polymerase chain reaction (PCR) with primers flanking the exon, as previously described.[39] For the reverse dot blot hybridization reaction, oligonucleotides specific for either the *Pl^A1* or *Pl^A2* allele were covalently attached to filters and hybridized with biotinylated PCR products of glycoprotein IIIa exon 2, and reactivity was assessed by an enhanced chemiluminescence technique, as described previously.[34,37] These allele-specific hybridization data were confirmed with restriction-enzyme digestion with *Msp*I and *Nci*I (New England Biolabs, Beverly, Mass.), which are able to distinguish the *Pl^A1* allele from the *Pl^A2* allele because new restriction sites are generated as the result of the *Pl^A2* polymorphism. Exon 2 PCR products were digested separately with both enzymes, and the resulting fragments were analyzed on a 3 percent agarose gel. The results of both techniques were confirmed by at least two independent investigators who were unaware of the origin of the DNA.

Statistical Analysis

The size of the sample was established after pilot studies indicated that the prevalence of *Pl^A2* among the case patients would be approximately 40 percent and the expected prevalence in control subjects would not exceed 20 percent. With the expectation that a difference of this magnitude would strongly support the concept that *Pl^A2* is a significant genetic risk factor, and with a one-sided alpha error of 0.05, the size of the sample was set at 71 to limit the beta error to 0.1. Student's t-test was used to compare established risk factors for coronary heart disease in the case patients and the control subjects, with a two-tailed test used for the continuous variables. A P value of 0.05 or less was considered to indicate statistical significance. Discrete data (including data on the *Pl^A* alleles) were analyzed by the chi-square test. The strength of the association of the *Pl^A2* genetic factor with the occurrence of acute coronary events was estimated by calculation of the odds ratios with EpiInfo software (version 6, Centers for Disease Control and Prevention, Atlanta) and the Cornfield method for the calculation of 95 percent confidence intervals. The relative strength of association of other risk factors was measured in a similar manner. The significance of the difference in the odds ratios was not tested, since the sample size was not designed for such analyses. The relation of the *Pl^A* allele to each of the remaining predictor variables was examined by bivariate chi-square analysis. Finally, the association of the *Pl^A2* allele with coronary events, standardized for the other risk factors, was determined by the multiple logistic-regression method with the Stata statistical package (version 4.0, Stata, College Station, Tex.).

RESULTS

Characteristics of the Study Population

Table 1 shows the prevalence of selected risk factors for coronary heart disease among the case patients and controls. The two groups were matched according to age, race, and sex, and there were therefore no significant differences in these variables. Owing to the sample size, the only risk factor that differed significantly between the groups was smoking status (P = 0.05).

Prevalence of *Pl^A2*

Genotyping results for 3 of the 71 case patients are shown in Figure 1, demonstrating the three possible *Pl^A* allelic combinations. Table 2 summarizes the genotyping data for all case patients and controls. The prevalence of *Pl^A2* among the case subjects was 39.4 percent (percentage of subjects who were either heterozygous [*Pl^A1/Pl^A2*] or homozygous [*Pl^A2/Pl^A2*]), a value that was significantly higher than the prevalence among the 68 controls (19.1 percent, P = 0.01). The association between *Pl^A2* and coronary events was even stronger in patients who were less than 60 years of age when they had their first coronary event. Of the 42 such patients, 50 percent carried at least one *Pl^A2* allele, as compared with 13.9 percent of the 36 controls who were less than 60 years of age (P = 0.002).

Comparison of Major Risk Factors for Coronary Heart Disease

Among the major risk factors for coronary heart disease examined in this study, the risk factor associated with the highest estimated odds ratio was carriage of the *Pl^A2* allele (odds ratio, 2.8; 95 percent confidence interval, 1.2 to 6.4), followed in order by smoking (odds ratio, 2.2; 95 percent confidence interval, 1.0 to 4.8), hypertension (systolic blood pressure, ≥140 mm Hg) (odds ratio, 1.9; 95 percent confidence interval, 0.9 to 3.9), and hypercholesterolemia (total serum cholesterol level, ≥200 mg per deciliter [5.2 mmol per liter]) (odds ratio, 1.3; 95 percent confidence interval, 0.5 to 3.0) (Table 3). The prevalence of diabetes mellitus was sim-

Table 1. Prevalence of Selected Risk Factors for Coronary Heart Disease among Case Patients and Controls.

RISK FACTOR	CASE PATIENTS (N = 71)	CONTROLS (N = 68)	P VALUE*
Age (yr)†	56.3±12.8	58.3±14.3	0.39
Male sex (%)	70	69	0.99
Hypertension (%)‡	52	37	0.10
Current or former smoker (%)	72	55	0.05
Hypercholesterolemia (%)§	26	22	0.69
Type I or II diabetes mellitus (%)	18	22	0.73

*Student's t-test (for continuous variables) and the chi-square test (for discrete variables) were used to compare the values for case patients and controls.

†Plus–minus values are means ±SD; for the case patients, the value refers to the age at onset of myocardial infarction or unstable angina, not the current age.

‡Hypertension was defined as a systolic blood pressure ≥140 mm Hg at the time of admission to the hospital.

§Hypercholesterolemia was defined as a total serum cholesterol level ≥200 mg per deciliter (5.2 mmol per liter) at the time of admission to the hospital.

Figure 1. Genotyping of PI^A Loci by Allele-Specific Restriction Digestion and Reverse Dot Blot Hybridization.

Panel A shows the restriction map of exon 2 of the glycoprotein IIIa gene, undigested fragments, and fragments resulting when PI^{A1} or PI^{A2} PCR products were digested with the restriction enzymes MspI and NciI. Sizes (in base pairs) are indicated below the horizontal lines. The vertical bars indicate the ends of PCR fragments or digestion products. For clarity and because it does not cause a detectable change in the fragment sizes, an additional MspI site (present in both PI^A alleles) 7 bp from the 3′ MspI site is not shown. Panel B shows a representative ethidium-stained 3 percent agarose gel containing undigested PCR products (lanes 1, 5, and 8) and PCR products digested with MspI (lanes 2, 6, and 9) and NciI (lanes 3, 7, and 10) corresponding to the three possible allelic combinations: PI^{A1}/PI^{A1} (lanes 1, 2, and 3), PI^{A1}/PI^{A2} (lanes 5, 6, and 7), and PI^{A2}/PI^{A2} (lanes 8, 9, and 10). Lane 4 shows ϕX174 DNA cut with HaeIII, which was used as a size marker. Panel C shows the results of reverse dot blot hybridization with the DNA shown in Panel B. Filters are shown with PI^{A1}-allele–specific oligonucleotides at the top and PI^{A2}-allele–specific oligonucleotides at the bottom.
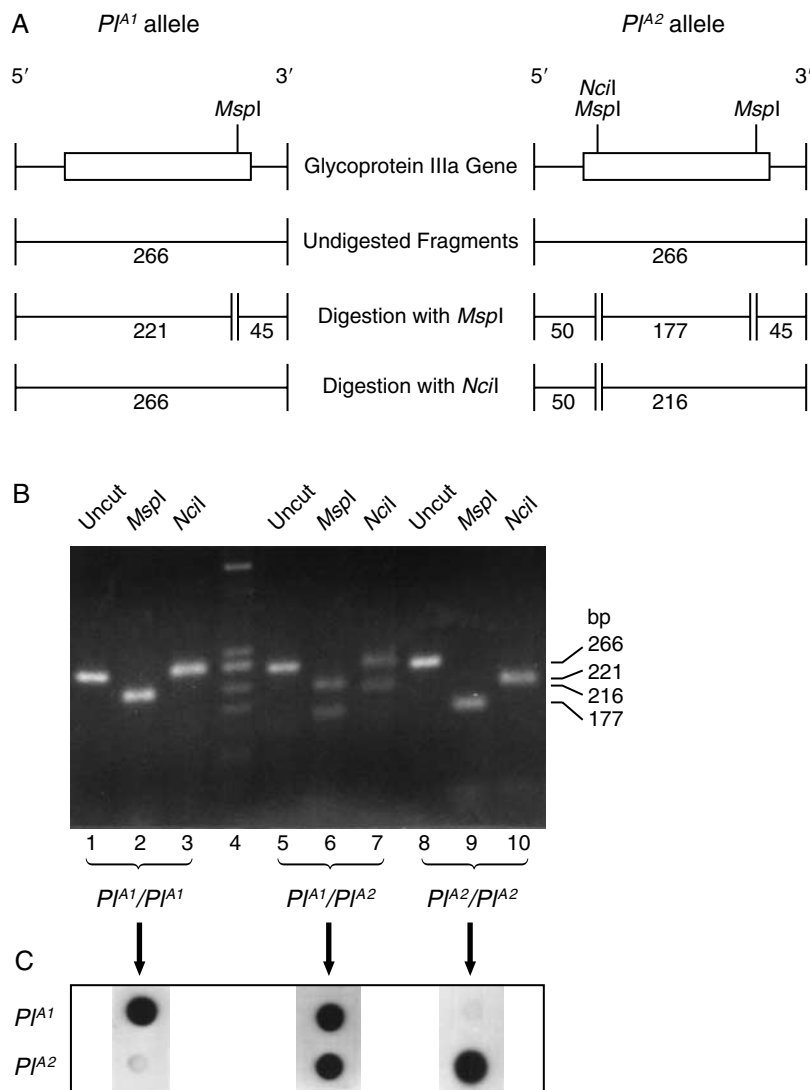
ilarly high among case patients and control subjects. As anticipated, the odds ratios for each of the risk factors, including PI^{A2}, were higher in the subgroup of case patients less than 60 years of age than in controls less than 60 years of age. In this subgroup, the risk factor associated with the highest estimated odds ratio was again the PI^{A2} allele (odds ratio, 6.2; 95 percent confidence interval, 1.8 to 22.4), followed in order by smoking (odds ratio, 3.8; 95 percent confidence interval, 1.2 to 12.0), hypercholesterolemia (odds ratio, 3.7; 95 percent confidence interval, 0.8 to 18.7), and hypertension (odds ratio, 2.1; 95 percent confidence interval, 0.7 to 5.9) (Table 3).

A multiple logistic-regression model that adjusted for the presence of smoking, hypertension, hypercholesterolemia, and an age greater than 60 years provided an odds ratio of 3.3 (95 percent confidence interval, 1.4 to 7.7) for the relation between acute coronary events and the PI^{A2} allele. This was consistent with the finding that the bivariate association of the allele with smoking, hy-

pertension, hypercholesterolemia, and an age greater than 60 years was uniformly not statistically significant (data not shown).

DISCUSSION

Our data demonstrate an association between the PI^{A2} polymorphism of glycoprotein IIIa and the occurrence of acute coronary thrombosis. We found a significantly higher prevalence of subjects with at least one PI^{A2} allele among those with either myocardial infarction or unstable angina than among a control group matched for sex, age, and race. If this polymorphism represents an inherited risk factor for myocardial infarction or unstable angina, one would predict an even higher prevalence of this risk factor among persons in whom these disorders occur at a younger age (before the age of 60 years), and this is what we found.

Data from northern and central Europe estimate the phenotypic frequency and genotypic frequency of PI^{A2} to be 26.5 percent and 15 percent, respectively.[40] These

Table 2. Genotypes of the Case Patients and Controls According to Age.*

GENOTYPE	CASE PATIENTS	CONTROLS	P VALUE†
	no. (%)		
All ages			
Pl^{A1}/Pl^{A1}	43 (60.6)	55 (80.9)	
$Pl^{A1}/Pl^{A2} + Pl^{A2}/Pl^{A2}$	23+5 (39.4)	12+1 (19.1)	0.01
Total	71 (100)	68 (100)	
Age <60 yr			
Pl^{A1}/Pl^{A1}	21 (50)	31 (86.1)	
$Pl^{A1}/Pl^{A2} + Pl^{A2}/Pl^{A2}$	19+2 (50)	5+0 (13.9)	0.002
Total	42 (100)	36 (100)	

*Subjects with the Pl^{A1}/Pl^{A1} genotype were classified as Pl^{A2}-negative, and subjects with the Pl^{A1}/Pl^{A2} or Pl^{A2}/Pl^{A2} genotype were classified as Pl^{A2}-positive. For the case patients, age refers to the age at onset of myocardial infarction or unstable angina, not the current age.

†The chi-square test was used to compare the prevalence of Pl^{A2} in case patients and controls.

estimates were derived from studying subjects whose genetic background may differ from that of our subjects. In our previous work, we determined that the prevalence of Pl^{A2} among 100 white subjects from the Baltimore area was 20 percent (genotypic frequency, 11 percent).[34] As compared with the subjects in the European studies, the control subjects in the current study had a slightly lower prevalence of Pl^{A2} (19.1 percent vs. 26.5 percent), but one that was virtually identical to the prevalence in our previous study in Baltimore. Between our two studies we have performed genotyping on 336 chromosomes from white control subjects, and the results provide very strong evidence that the prevalence of Pl^{A2} among these subjects in the metropolitan Baltimore area is approximately 20 percent. For this reason, we believe the data on control subjects in the current study accurately reflect the population genetics in our geographic area and that this group is a valid one to use for comparison with our case patients.

Finally, although variation in the genetic background is the most likely explanation for differences in the prevalence of the Pl^{A2} phenotype between the United States and Europe, the earlier studies of European pop-

ulations used immunophenotyping, which is less specific than genotyping.

To assess the association between Pl^{A2} and acute coronary events, we compared the risk of having a coronary event associated with the Pl^{A2} polymorphism with that associated with four major cardiac risk factors. Among the major risk factors tested, the one associated with the largest odds ratio of having a coronary event, both for the entire group and for the subgroup of younger patients (age less than 60 years at first acute coronary event), was the Pl^{A2} allele (Table 3). In addition, we conducted a multiple logistic-regression analysis adjusting for the presence of smoking, hypertension, hypercholesterolemia, and an age greater than 60 years, which demonstrated an independent association between Pl^{A2} and acute coronary events. All the risk factors tested yielded odds ratios consistent with what one would expect for a major risk factor for coronary heart disease, except for diabetes. However, we believe that the low odds ratio associated with diabetes is a result of the selection of control subjects from a population of inpatients with a high prevalence of diabetes.

One theoretical explanation for the increased prevalence of Pl^{A2} among the case patients could be a higher survival rate after myocardial infarction in this group than in Pl^{A2}-negative patients. However, if Pl^{A2} did provide a survival advantage, the prevalence of Pl^{A2} would be unlikely to be increased among the case patients in whom disease occurred before the age of 60 years. Instead, our hypothesis is that Pl^{A2} is a risk factor for coronary heart disease events, particularly among younger persons. In fact, among the case patients, there was a significant difference of more than 7 years in the age at onset of disease between the Pl^{A2}-positive patients and the Pl^{A2}-negative patients (51.8 vs. 59.2 years, P=0.02).

In addition to our findings related to the prognostic potential of Pl^{A2}, our results could directly affect the treatment of acute coronary events. For example, although aspirin has been the platelet inhibitor of choice for the treatment of unstable coronary syndromes, re-

Table 3. Odds Ratios for Selected Risk Factors, According to Age.*

FACTOR	CLASS	ALL AGES			AGE <60 YR		
		NO. OF CASE PATIENTS	NO. OF CONTROLS	ODDS RATIO (95% CI)	NO. OF CASE PATIENTS	NO. OF CONTROLS	ODDS RATIO (95% CI)
Pl^A genotype	Pl^{A2}-positive†	28	13	2.8 (1.2–6.4)	21	5	6.2 (1.8–22.4)
	Pl^{A2}-negative	43	55		21	31	
Smoking status	Current or former	50	36	2.2 (1.0–4.8)	32	18	3.8 (1.2–12.0)
	Never	19	30		8	17	
Systolic blood pressure on admission	≥140 mm Hg	36	25	1.9 (0.9–3.9)	19	11	2.1 (0.7–5.9)
	<140 mm Hg	33	43		21	25	
Total serum cholesterol on admission	≥200 mg/dl	18	15	1.3 (0.5–3.0)	10	3	3.7 (0.8–18.7)
	<200 mg/dl	50	53		30	33	
Diabetes mellitus	Type I or II	13	15	—‡	8	8	—‡
	None	58	53		34	28	

*The total number of patients and controls for individual risk factors may not equal 71 and 68, respectively, since data were missing for some subjects. For the case patients, age refers to the age at onset of myocardial infarction or unstable angina, not the current age. CI denotes confidence interval.

†Subjects with the Pl^{A1}/Pl^{A2} or Pl^{A2}/Pl^{A2} genotype were classified as Pl^{A2}-positive.

‡The odds ratio was not calculated because the control subjects were selected from a population of inpatients with a high prevalence of diabetes.

cent studies indicate that treatment with specific inhibitors of glycoprotein IIb/IIIa leads to better outcomes than does aspirin therapy.[23-26,41] It is conceivable that Pl^{A2}-positive patients would receive extra benefit from direct therapy with anti–glycoprotein IIb/IIIa, providing a rationale for decisions regarding the choice of antiplatelet therapy for patients with unstable coronary syndromes.[41]

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